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GABA Accumulation Causes Cell Elongation Defects and a Decrease in Expression of Genes Encoding Secreted and Cell Wall-Related Proteins in *Arabidopsis thaliana*

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GABA (γ-aminobutyric acid), a non-protein amino acid, is a signaling factor in many organisms. In plants, GABA is known to accumulate under a variety of stresses. However, the consequence of GABA accumulation, especially in vegetative tissues, remains poorly understood. Moreover, gene expression changes as a consequence of GABA accumulation in plants are largely unknown. The *pop2* mutant, which is defective in GABA catabolism and accumulates GABA, is a good model to examine the effects of GABA accumulation on plant development. Here, we show that the *pop2* mutants have pollen tube elongation defects in the transmitting tract of pistils. Additionally, we observed growth inhibition of primary root and dark-grown hypocotyl, at least in part due to cell elongation defects, upon exposure to exogenous GABA. Microarray analysis of *pop2-1* seedlings grown in GABA-supplemented medium revealed that 60% of genes whose expression decreased encode secreted proteins. Besides, functional classification of genes with decreased expression in the *pop2-1* mutant showed that cell wall-related genes were significantly enriched in the microarray data set, consistent with the cell elongation defects observed in *pop2* mutants. Our study identifies cell elongation defects caused by GABA accumulation in both reproductive and vegetative tissues. Additionally, our results show that genes that encode secreted and cell wall-related proteins may mediate some of the effects of GABA accumulation. The potential function of GABA as a growth control factor under stressful conditions is discussed.

Keywords: *Arabidopsis thaliana* • Cell elongation • Cell wall • GABA • Microarray • Secretory pathway.

Abbreviations: AABA, α-aminobutyric acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AGP, arabinogalactan protein; BABA, β-aminobutyric acid; DIC, differential interference contrast; FT-IR, Fourier transform-infrared; GABA, γ-aminobutyric acid; GST, gene-specific tag; GUS, β-glucuronidase; qRT-PCR, quantitative real-time PCR

Introduction

GABA (γ-aminobutyric acid) is a ubiquitous non-protein amino acid, found in prokaryotes and eukaryotes (for a review, see Bouché et al. 2003). Metabolism of GABA takes place in two cellular compartments; GABA synthesis occurs in the cytosol whereas GABA is degraded in the mitochondrion (**Supplementary Fig. S1**). Initially discovered in plants (Steward et al. 1949), GABA has received increased attention in mammals due to its effects on signaling and development; it is now well documented that GABA is a key component of nervous signal transduction and brain network establishment (Owens and Kriegstein 2002, Represa and Ben-Ari 2005). Moreover, GABA is known to impact development of other organisms such as planktonic larvae (Morse et al. 1979) and bacteria (Foerster 1971, Chevrot et al. 2006). In plants, GABA has been mostly investigated as a metabolite and is thought to function in anaplerotic alimentation of the tricarboxylic acid (TCA) cycle and C/N balance control (reviewed in Fait et al. 2008). In contrast, the role of GABA in plant development, particularly vegetative development, has received little attention, even though several studies have reported on its rapid accumulation in response to environmental cues (Kinnersley and Turano 2000). As a result, the consequences of GABA accumulation in development remain unclarified (Bouché and Fromm 2004). However, it appears that GABA is important

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for vegetative development; overexpression of a hypermorphic glutamate decarboxylase, the enzyme that catalyzes the conversion of glutamate to GABA (Supplementary Fig. S1), led to an alteration of tobacco vegetative development (Baum et al. 1996).

In the mammalian nervous system, GABA is the major inhibitory neurotransmitter (Pinal and Tobin 1998). Moreover, it also functions as a trophic factor during early brain development, and can promote polarized growth of neurites in the cerebellum (Pinal and Tobin 1998). The guidance role of GABA is concentration dependent; GABA directs migration of post-mitotic neuroblasts at femtomolar (10^{-15} M) concentrations. In contrast, at micromolar concentrations, GABA stimulates random motility via Ca^{2+} signaling mechanisms (Barker et al. 1998). Previous research indicates that GABA may also play a guidance role in plants. Analysis of Arabidopsis *pop2* mutants, which are impaired in the first step of GABA degradation (*pop2* mutants; Supplementary Fig. S1), showed a very similar phenomenon: flowers with low levels of GABA (200 μM) display normal guidance, while increases 100-fold above that level cause severe defects in guidance in vivo and cause decreased pollen tube growth in vitro (Palanivelu et al. 2003). Finally, it was also shown that *pop2* mutants accumulated much more GABA in flowers than leaves and, even though the floral phenotype (sterility) was readily detected, no obvious vegetative phenotypes were observed (Palanivelu et al. 2003).

In mammalian systems, interaction of GABA with the receptor required for polarized neurite growth ($GABA_B$) results in the direct activation of transcription factors that in turn cause changes in downstream gene expression (Nehring et al. 2000, White et al. 2000). Likewise, in sunflower, treatment of excised cotyledons with GABA stimulates ethylene production through the induction of genes involved in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Kathiresan et al. 1997). GABA is also known to modulate nitrate influx in *Brassica napus* (Beuve et al. 2004) and is involved in the decrease in expression of several *14-3-3* genes in *Arabidopsis thaliana* (Lancien and Roberts 2006). Furthermore, Roberts (2007) first reported on the impact of exogenous GABA on the transcriptome of Arabidopsis wild-type plants. However, that study did not focus on the identity of genes deregulated in response to GABA, a subject that was examined in this study.

Here we report that besides the pollen tube guidance defects, *pop2* pollen tube elongation is also inhibited in vivo. We also investigated vegetative phenotypes in *pop2* mutants and found that, when grown in GABA-supplemented medium, *pop2* seedlings exhibited primary root growth and hypocotyl elongation defects. To gain insights into the determinants underlying these GABA-dependent responses, we combined physiological, cellular and comparative microarray analyses and identified a negative impact of GABA on cell elongation.

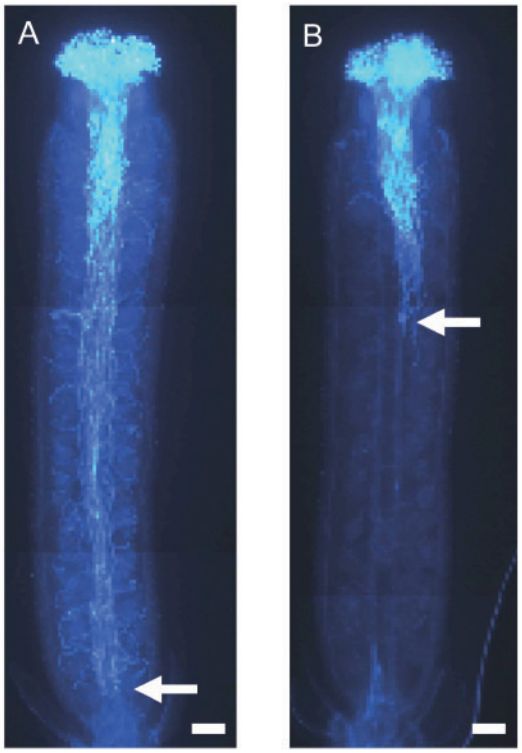
Results

pop2-1 pollen tubes fail to elongate normally in *pop2-1* pistils

Previously, a role for GABA in pollen tube guidance had been described (Wilhelmi and Preuss 1996, Palanivelu et al. 2003). Additionally, it was reported that exogenous GABA levels influenced pollen tube growth in vitro; although GABA stimulates pollen tube growth at lower concentrations, much higher concentrations inhibited tube elongation in vitro (Palanivelu et al. 2003). However, in vivo pollen tube growth in *pop2-1* pistils was not examined. Here, we monitored pollen tube growth in self-pollinated *pop2-1/pop2-1* pistils, that have been shown to accumulate high levels (>1,700 μM) of GABA (Palanivelu et al. 2003). We observed that *pop2-1* mutant tubes, unlike a wild-type pollen tube in a wild-type pistil (Fig. 1A), did not traverse the entire length of a *pop2-1/pop2-1* pistil and failed to elongate beyond the middle point of a *pop2-1/pop2-1* pistil (Fig. 1B). The observed elongation defect was terminal as pollen tubes did not elongate any further in a pistil even when observed 36 h after pollination (data not shown). As reported for other *pop2-1* reproductive defects (Palanivelu et al. 2003), the pollen tube elongation defect is also self-sterile in nature; the defect manifested only when mutant pollen tubes elongated through a mutant pistil but not when a wild-type tube elongated through a *pop2-1/pop2-1* pistil or vice versa (data not shown).

Exogenous GABA results in an increase in shoot and root GABA levels

Previously we had shown that *pop2-1* contained higher GABA levels, 113- and 23-fold higher in mutant flowers and leaves, respectively, compared with wild-type flowers and leaves (Palanivelu et al. 2003). Although *pop2-1/pop2-1* flowers were sterile, no obvious defects were detected in vegetative tissues. We hypothesized that if GABA levels in *pop2-1* vegetative tissues approach the high levels detected in mutant flowers, vegetative tissues might exhibit detectable developmental defects. Therefore, we attempted to increase GABA levels in *pop2* mutant vegetative tissues. Since exposure to exogenous GABA increases intracellular GABA levels (Kathiresan et al. 1997, Beuve et al. 2004), we grew *pop2* seedlings on medium supplemented with 1 mM GABA, as this concentration was close to the GABA levels reported in *pop2-1/pop2-1* pistils (Palanivelu et al. 2003). We transferred 10-day-old seedlings onto medium with and without GABA and, after 4 d, measured GABA levels. Compared with seedlings that grew on medium without GABA, a higher GABA level was detected in roots and shoots of the wild type and the two *pop2* mutants that grew on medium with GABA (Table 1). Moreover, both *pop2* mutants contained higher steady-state GABA levels in shoots and roots than the corresponding wild-type controls (Table 1). We identified no changes in glutamate decarboxylase (GAD) and GABA transaminase (GABA-T) activities (Supplementary Table S1)



C

Female parent	Male parent	Pollen tube front in a pistil[a] (mm)	Number of pistils scored[b]
POP2/POP2	*POP2/POP2*	0.38 ± 0.02	28
pop2-1/pop2-1	*pop2-1/pop2-1*	0.17 ± 0.04	39

[a]Distance between stigma and pollen tube front (for example, white arrows in Figures 1A, B)
[b]Pistils were stained with aniline blue to visualize pollen tube growth 18 hours after pollination

Fig. 1 Pollen tube growth and guidance in pistils. (A, B) Aniline blue-stained pollen tubes in self-pollinated wild-type (A) and *pop2-1/pop2-1* (B) pistils. White arrows point to the pollen tube growth front in the pistils. (C) A table summarizing pollen tube growth in wild-type and *pop2-1/pop2-1* siliques. Scale bar, 50 μm.

Table 1 GABA levels in seedlings after exposure to exogenous GABA

Genotype	GABA content (μmol g^{-1} DW)			
	Control		1 mM GABA	
	Roots	Shoots	Roots	Shoots
Ler	5.8 ± 0.40	0.6 ± 0.04	16.2 ± 0.31	1.7 ± 0.13
pop2-1	13.9 ± 0.34	11.9 ± 0.17	182.2 ± 8.12	129.9 ± 0.94
pop2-1/*Ler*	2.4	20	11	76
Col	6.0 ± 0.49	0.6 ± 0.05	12.5 ± 0.56	1.4 ± 0.12
pop2-8	13.6 ± 0.35	10.7 ± 0.15	79.5 ± 1.75	90.2 ± 2.30
pop2-8/Col	2.3	18	6.4	64

GABA levels were determined in roots and shoots of 14-day-old seedlings treated for 4 d with (1 mM) or without (control) GABA. Results are the mean ± SE of three independent experiments.

in wild-type seedlings exposed to 1 mM GABA for 4 d, indicating that the increases in GABA level observed in wild-type shoots and roots were primarily due to exogenous GABA rather than a consequence of alterations in intracellular GABA metabolism.

Exogenous GABA induces chlorosis in *pop2* mutant leaves

We next examined whether the increased in vivo GABA levels from exogenous GABA caused any alterations in shoots. In wild-type seedlings, exogenous GABA did not result in any obvious changes in shoots (Fig. 2A). However, in *pop2-1* and *pop2-8* there was a noticeable yellowing of aerial parts (Fig. 2B). Consistent with these results, we observed a significant

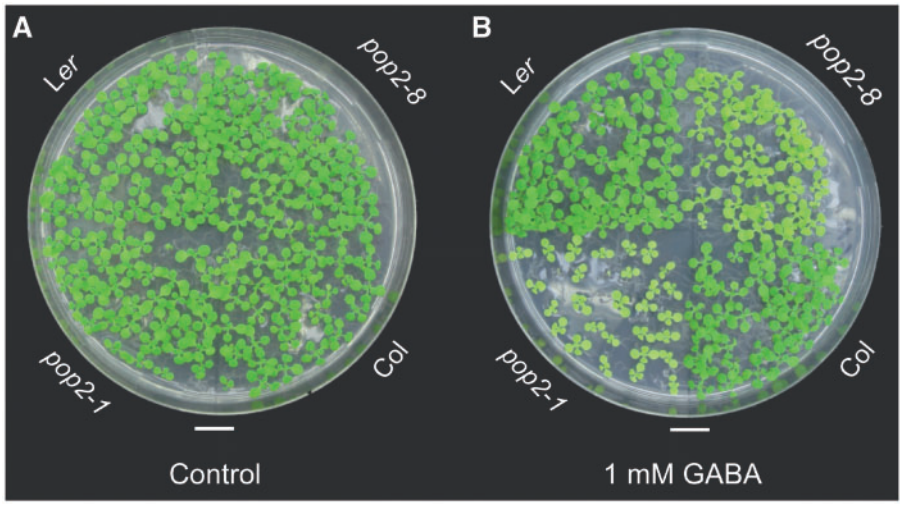


Fig. 2 Leaf phenotype of *pop2* mutants grown on GABA-supplemented medium. Photographs of *pop2* mutants and corresponding wild-type ecotypes grown for 2 weeks on agar medium supplemented with no (control, A) or 1 mM GABA (B). Scale bar, 1 cm.

decrease in both the total Chl and carotenoid content of *pop2* leaves, whether they were grown in medium supplemented with 1 or 10 mM GABA (**Supplementary Fig. S2A, B**). In wild-type leaves, 1 mM exogenous GABA did not result in a significant change in content of photosynthetic pigments (**Supplementary Fig. S2A, B**), consistent with the lack of yellowing of aerial parts in wild-type plants (**Fig. 2B**). However, in wild-type plants grown on 10 mM GABA-supplemented medium, we observed a modest, yet significant, increase in photosynthetic pigment levels (**Supplementary Fig. S2A, B**).

Exogenous GABA inhibits primary root growth in *pop2* mutants

After examining shoots, we next tested the impact of exogenous GABA on primary root growth by transferring 4-day-old seedlings to agar plates supplemented with GABA, then recording primary root length after 6 d. The primary root length of wild-type plants grown on plates without GABA was indistinguishable from that of those grown on plates supplemented with 1 mM GABA (**Fig. 3A**). However, when exposed to 10 mM GABA, 24 and 21% inhibition in primary root growth of wild-type Landsberg *erecta* (*Ler*) and Columbia (Col) plants, respectively, was observed (**Fig. 3B**), consistent with the previously reported results (Roberts 2007). In the case of *pop2* plants grown with exogenous GABA compared with (i) those mutant plants grown on plates without GABA and (ii) the wild-type plants grown on plates with or without GABA, we observed a noticeable decrease in primary root length (**Fig. 3A**). Indeed, 1 and 10 mM GABA caused 54 and 86% inhibition of primary root growth in *pop2-1* plants compared with *pop2-1* plants grown without GABA (**Fig. 3B**). A similar inhibition of primary root growth was also observed in response to 1 mM GABA in *pop2-3* and *pop2-8* mutants (42 and 31%). These results suggest that primary root growth is sensitive to exogenous GABA and that this phenotype is more pronounced in *pop2* mutants. Notably, the decrease in growth of *pop2* primary root in response to exogenous GABA is reminiscent of the *pop2-1* pollen tube elongation defects in a *pop2-1*/*pop2-1* pistil (**Fig. 1B**).

To test whether the decrease in primary root length was due to a decrease in root cell length, we examined wild-type and *pop2-1* roots after 6 d of treatment with 1 mM GABA (the phenotype of plants is visible by this time as shown in **Fig. 3A**) by confocal microscopy. We imaged root cells at the beginning of the root hair zone, as it is the region where cells have completed lengthening and are in the maximum elongated state. While 1 mM GABA treatment did not result in any significant change in size of cortical cells, chosen as a representative cell type, in the wild type compared with control conditions (**Fig. 3C, D**), we observed a severe and significant inhibition of cell elongation in the *pop2-1* mutant; cortical cell size was decreased by as much as 37%, compared with those in roots grown in control conditions (**Fig. 3D, E**). These results indicate that the inhibitory effect of GABA on primary root growth was, at least in part, due to an inhibition of cortical cell elongation.

The specificity of the primary root growth response to GABA was tested by supplementing the growth medium with 1 mM of other forms of aminobutyric acid [β-aminobutyric acid (BABA) and α-aminobutyric acid (AABA)], or compounds resulting from GABA catabolism (i.e. alanine, succinate and γ-hydroxybutyric acid; **Supplementary Fig. S1**). Glutamine, a neutral amino acid, was chosen as a control. None of these compounds, except GABA, impacted differentially on primary root growth of the *pop2-1* mutants (**Supplementary Fig. S3**). Other aminobutyric acids (i.e. BABA and AABA) were found to be deleterious for primary root growth of *A. thaliana*, suggesting that GABA is the non-toxic form of aminobutyric acid (**Supplementary Fig. S3**).

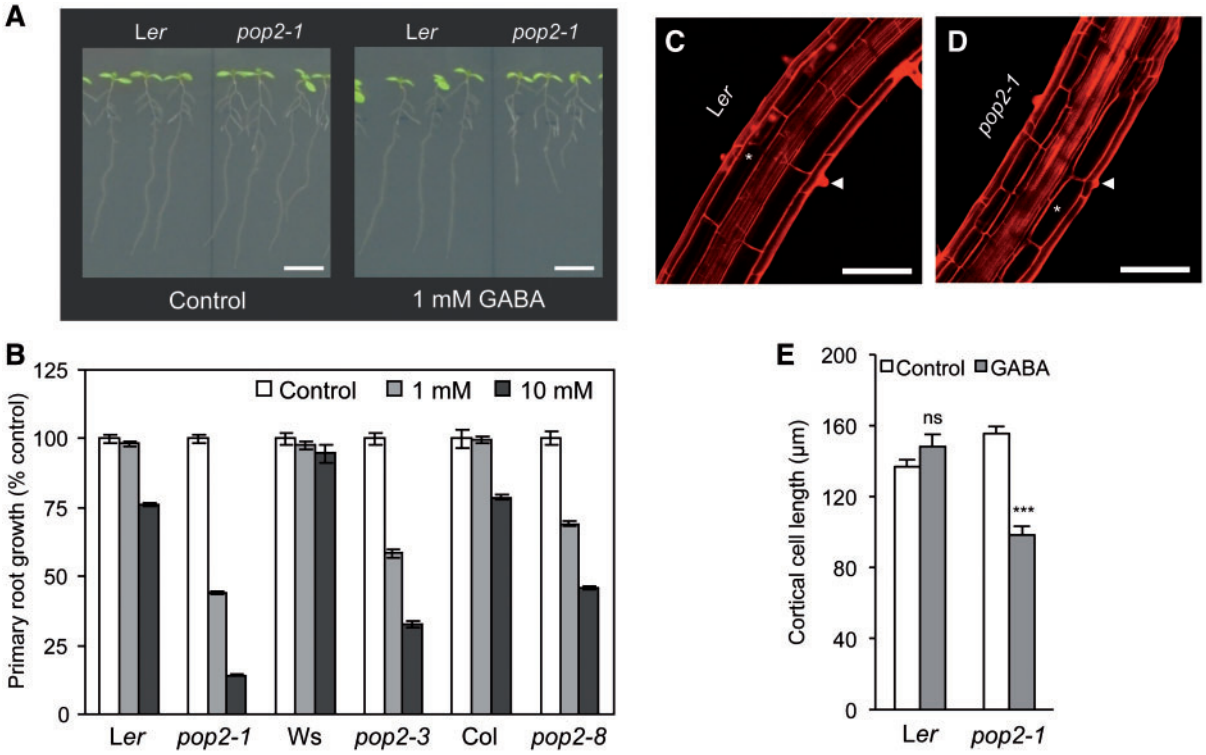


Fig. 3 Root phenotype of *pop2* mutants grown on GABA-supplemented medium. Four-day-old seedlings grown on agar plates were transferred to fresh agar medium supplemented with GABA. Six days after transfer, primary root growth was evaluated. (A) Photographs of root growth in the indicated *pop2* mutants or their corresponding wild-type strains. Scale bar, 1 cm. (B) Primary root growth of the indicated *pop2* mutants and the corresponding wild-type strains on the indicated concentrations of GABA-supplemented medium. Results presented are the mean ± SE of measurements made on 16–18 plants distributed over three plates. (C–E) Confocal images of roots treated with GABA. Typical images of wild-type (C) and *pop2-1* (D) roots after treatment with 1 mM GABA for 6 d are shown. Asterisks indicate a typical cortical cell. Arrowheads point to emerging root hairs. Scale bar, 100 µm. (E) Average root cortical cell size. Results are the mean ± SE of 10–45 cells derived from at least five different roots. Student's *t*-test: ns, not significant; ***$P < 0.001$.

Transcriptome analysis reveals a decrease in expression of genes encoding cell wall-related and secreted proteins in *pop2-1* plants

Despite the preliminary work of Roberts (2007) and a few other studies (Kathiresan et al. 1997, Beuve et al. 2004, Lancien and Roberts, 2006) showing that GABA could modulate gene expression, a comprehensive study of gene expression changes in vegetative tissues upon GABA accumulation or exogenous application of GABA has not been performed. Consequently, the genes that underlie the plant response to high GABA levels, including the vegetative and reproductive phenotypes described here, remain unknown. We reasoned that comparing the transcriptomes of wild-type and *pop2* plants will identify genes underlying the *pop2-1* phenotypes detected when grown in the presence of exogenous GABA.

Unlike flowers, the vegetative tissues of *pop2* mutants did not exhibit any obvious phenotypes under standard growth conditions (**Figs. 2**, **3**), perhaps due to lower GABA levels in leaves of *pop2* mutants relative to flowers (Palanivelu et al. 2003). We therefore analyzed transcriptomes of *pop2-1* seedlings after treatment without or with 1 mM exogenous GABA, a condition in which mutant seedlings accumulated high levels of GABA and exhibited shoot and root phenotypes (**Table 1**, **Figs. 2**, **3**). The microarray strategy relied on the comparative analysis of wild-type and *pop2-1* transcriptomes, allowing us to identify genes differentially expressed in the mutant. Three time points were considered for microarray analyses of GABA-treated seedlings: time 0 (no treatment), 1 d of treatment (day 1) and 4 d of treatment (day 4); an overview of the experimental design (**Fig. 4A**), as well as the phenotype (**Fig. 4B**) and GABA content (**Fig. 4C**) of the plants prior to RNA extraction is presented in **Fig. 4**. Global analysis of the vegetative tissue transcriptome was carried out using the CATMA microarray (Crowe et al. 2003, Hilson et al. 2004) on total RNA isolated from whole seedlings. For each condition, two independent experiments were analyzed and technical reliability was assessed by performing one dye swap per biological replicate (i.e. a total of four slides for each condition). Only genes showing a statistically significant different expression level (Bonferroni *P*-value <0.05) between the wild type and *pop2-1* were considered for subsequent analysis (see the Materials and Methods for statistical analysis).

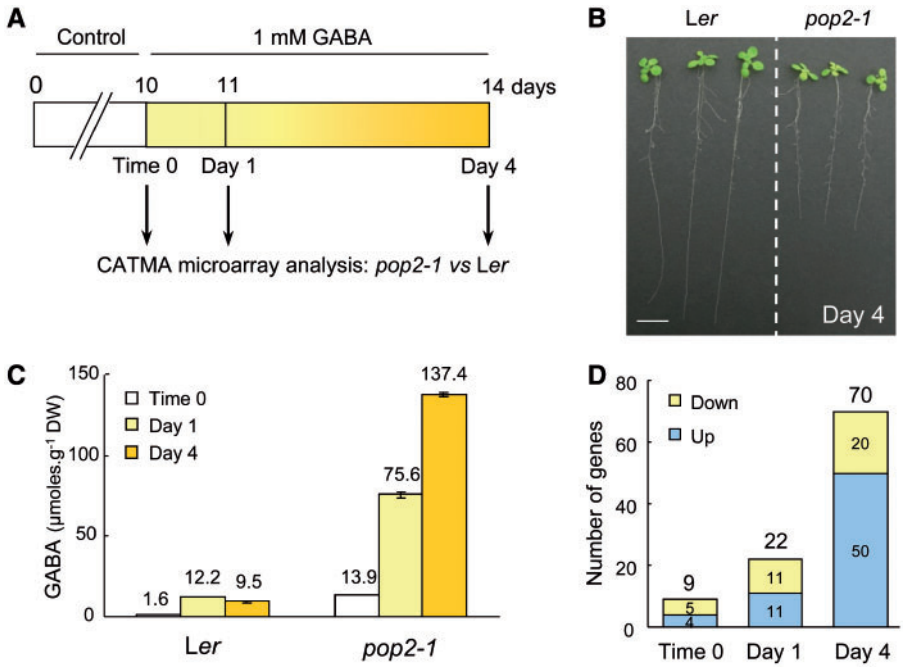


Fig. 4 Overview of the experimental design and results of CATMA microarray analysis. (A) Schematic representation of the experimental design. Ten-day-old wild-type and *pop2-1* mutant seedlings were transferred on agar medium supplemented with 1 mM GABA. Plants were harvested just before transfer (time 0), after 1 d (Day 1) and 4 d (Day 4) of treatment. Analysis consisted of comparison of *pop2-1* and wild-type transcriptomes at each time point. (B) The phenotype of plants on Day 4. Scale bar, 1 cm. (C) GABA content in whole seedlings at each time point. Results are the mean ± SE of three independent replicates. (D) Number of genes down- and up-regulated in the *pop2-1* mutant. Genes were considered to be differentially regulated when the *P*-value was <0.05 after Bonferroni correction.

The wild-type and *pop2-1* vegetative transcriptomes were comparable under standard growth conditions (no treatment, time 0), since only nine genes were significantly differentially expressed in the *pop2-1* compared with the wild type (**Fig. 4D**; the complete list is given in **Supplementary Dataset S1**). After 1 and 4 d of treatment with GABA, expression of 22 and 70 genes, respectively, was altered significantly in *pop2-1* compared with the wild type (**Fig. 4D**; the complete list is given in **Supplementary Dataset S1**). We used quantitative real-time PCR (qRT-PCR) to verify changes in gene expression data obtained from CATMA microarray experiments (**Supplementary Table S2**). Two genes with a higher expression level in *pop2-1* compared with the wild type, including the one that showed the largest increase in expression in *pop2-1* (ATPase, *At2g18193*), exhibited a similar pattern of change in qRT-PCR experiments (**Supplementary Table S2**). Four genes whose expression is lower in *pop2-1* compared with the wild type, including the one with the largest decrease (AGP30, *At2g33790*), also showed a similar pattern of decrease in qRT-PCR experiments (**Supplementary Table S2**). Based on these results, we conclude that confidence can be placed on the changes in gene expression in *pop2-1* identified by microarray experiments reported in this study (**Fig. 4**; **Supplementary Dataset S1**).

Functional classification of genes uniquely decreased in expression in *pop2-1* seedlings compared with the wild type after GABA treatment (i.e. non-redundant with those of the control condition; lists of genes used can be seen in **Fig. 5** and **Supplementary Table S3**) identified a set of genes involved in cell wall functions (**Table 2**). This decrease in expression of cell wall-related genes is consistent with the cell elongation defects underlying primary root growth inhibition in *pop2* mutants (**Fig. 3**). Strikingly, we found that more than half (16/26) of the genes in *pop2-1* seedlings whose expression is lower after treatment with GABA (**Fig. 5**) encoded secreted proteins. Together, these results suggest that genes encoding cell wall and secreted proteins are important components that underlie the *pop2-1* phenotypes in vegetative tissues that manifest when grown in the presence of exogenous GABA.

GABA inhibits hypocotyl elongation of dark-grown *pop2-1* plants

Microarray analyses revealed that cell wall-related gene expression is reduced in vegetative tissues of *pop2* plants compared with the wild type. Consistent with these observations, *pop2* plants exhibited elongation defects in pollen tubes (**Fig. 1**) and primary roots (**Fig. 3**), which are, at least in part, due to cell elongation defects. In *pop2* plants, we investigated if defects might also occur in etiolation, another developmental process that depends on cell elongation and cell wall modifications. We assessed whether high levels of GABA impacted etiolation by monitoring dark-grown hypocotyls in which cell elongation is

Locus	Description	Subcellular localization	*pop2-1*/*Ler* ratio (log2)		
			Time 0	Day 1	Day 4
At4g26010	peroxidase, putative	Secr. pathway	0.00	-0.63	0.21
At5g53250	AGP22 (ARABINOGALACTAN PROTEIN 22)	Secr. pathway	-0.04	-0.72	-0.45
At5g05270	chalcone-flavanone isomerase family	no targeting	-0.20	-0.60	-0.52
At5g54370	late embryogenesis abundant protein-related	Secr. pathway	-0.09	-0.73	-0.53
At2g39530	integral membrane protein, putative	Secr. pathway	0.01	-0.65	-0.57
At3g48940	remorin family	no targeting	0.25	-0.75	-0.58
At5g08640	FLS (FLAVONOL SYNTHASE)	no targeting	-0.03	-0.60	-0.60
At5g10130	pollen Ole e 1 allergen and extensin family	Secr. pathway	-0.17	-0.60	-0.61
At1g26770	AtEXPA10 (Arabidopsis thaliana EXPANSIN A10)	Secr. pathway	-0.13	-0.33	-0.61
At1g52400	BGL1 (BETA-GLUCOSIDASE HOMOLOG 1)	Secr. pathway	-0.16	-0.57	-0.62
At1g20070	unknown protein	Chloroplast	0.16	-0.42	-0.64
AtCg00480	ATP synthase CF1 beta chain	-	0.08	-0.52	-0.65
At1g67750	pectate lyase family	Secr. pathway	-0.06	-0.01	-0.69
At2g03090	AtEXPA15 (Arabidopsis thaliana EXPANSIN A15)	Secr. pathway	-0.37	-0.36	-0.72
At4g39800	MI-1-P SYNTHASE (Myo-inositol-1-phosphate synthase)	no targeting	0.17	-0.19	-0.73
At1g52820	2-oxoglutarate-dependent dioxygenase, putative	no targeting	-0.51	-0.26	-0.73
At3g45140	LOX2 (LIPOXYGENASE 2)	Chloroplast	-0.19	-0.34	-0.74
At3g18200	nodulin MtN21 family	Secr. pathway	0.02	-0.67	-0.74
At2g42250	CYP712A1 (cytochrome P450)	Secr. pathway	-0.23	-0.21	-0.74
At1g24020	MLP423 (MLP-LIKE PROTEIN 423)	no targeting	-0.26	-0.03	-0.75
At2g37180	RD28 (plasma membrane intrinsic protein 2;3)	Secr. pathway	-0.03	-0.40	-0.75
At4g17340	DELTA-TIP2/TIP2;2 (tonoplast intrinsic protein 2;2)	Secr. pathway	-0.07	-0.28	-0.76
At4g27290	S-locus protein kinase, putative	Secr. pathway	-0.15	-0.34	-0.78
At1g69530	AtEXPA1 (Arabidopsis thaliana EXPANSIN A1)	Secr. pathway	-0.16	-0.56	-0.83
At5g13930	AtCHS/CHS/TT4 (CHALCONE SYNTHASE)	no targeting	-0.05	-0.55	-0.92
At2g33790	AGP30 (ARABINOGALACTAN PROTEIN 30)	Secr. pathway	-0.44	-1.90	-1.98

Fig. 5 List of genes decreased in expression in the *pop2-1* mutant after GABA treatment. A negative ratio indicates that the gene is decreased in expression in the *pop2-1* mutant; a positive ratio indicates that the gene is increased in expression in the *pop2-1* mutant. Ratios in black boxes were not found to be statistically significant after Bonferroni correction ($P > 0.05$). For each locus, the subcellular localization of the corresponding protein is indicated according to data available on the Aramemnon web site (http://aramemnon.botanik.uni-koeln.de/).

Table 2 Functional classification of genes whose expression is significantly altered in the *pop2-1* mutant after GABA treatment

MapMan terms	Frequency[a]	Elements[b]	*P*-value
Increased in expression (47 genes)			
Biodegradation of xenobiotics	25.46	1	0.038
Miscellaneous	4.91	11	8.345e-06
Hormone metabolism	4.00	3	0.032
Decreased in expression (26 genes)			
Cell wall	12.02	5	4.829e-05
Secondary metabolism	8.74	3	4.373e-03
Not assigned	0.21	2	1.169e-03

Only significant terms (*P*-value < 0.05) are shown. Functional classification of genes was performed using the Classification SuperViewer on-line software (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) with MapMan terms. Input consisted of the loci found to be uniquely differentially expressed in the *pop2-1* mutant after GABA treatment (i.e. non redundant with control condition: **Fig. 5**, **Supplementary Table S3**).

[a] Frequency is normalized to the overall ID number within the Arabidopsis genome.

[b] Number of genes matching the term.

more pronounced (Gendreau et al. 1997) and is amenable to the use of Fourier transform-infrared (FT-IR) microspectroscopy to conduct cell wall fingerprinting. We demonstrated that exogenous GABA inhibited hypocotyl growth in *pop2* mutants in a dose-dependent manner (**Fig. 6A, B**). In wild-type plants, except for L*er* in which hypocotyl growth was weakly but gradually inhibited by increasing concentrations of GABA, there was no effect on hypocotyl growth by increasing concentrations of GABA up to 10 mM (**Fig. 6B**). Beyond the 10 mM concentration, exogenous GABA significantly inhibited hypocotyl growth, by 27, 8 and 14% in L*er*, Wassilewskija (Ws) and Col ecotypes, respectively.

We next tested if organ growth inhibition was due to cell elongation defects by fixing hypocotyls and observing them using differential interference contrast (DIC) microscopy. We demonstrated that epidermal cells in *pop2-1* dark-grown hypocotyls raised on a medium supplemented with 10 mM GABA were smaller than the epidermal cells in plants grown under control conditions (**Fig. 5D, E**); no significant changes were

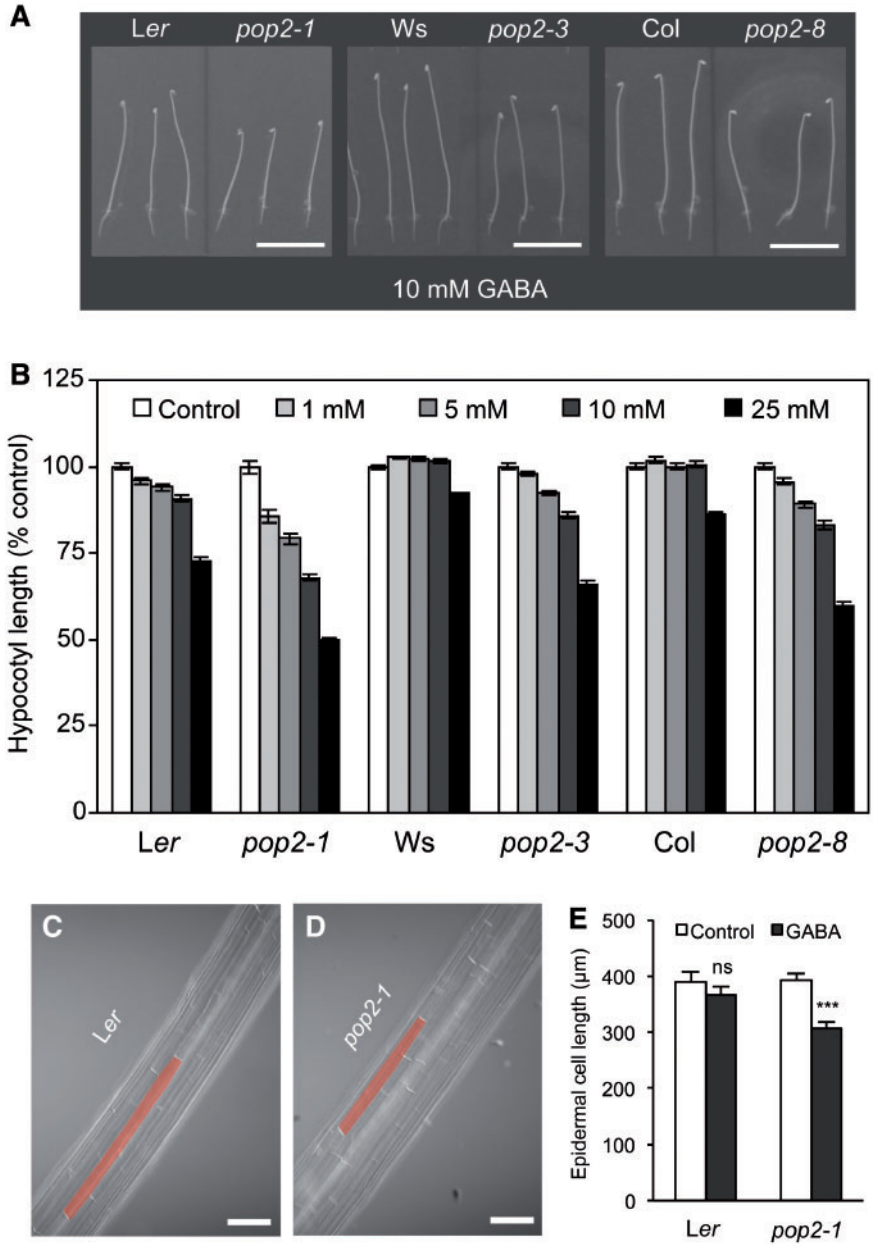


Fig. 6 Inhibition of hypocotyl growth in *pop2* mutants grown on GABA-supplemented medium. (A) Photographs of 4-day-old etiolated seedlings grown on 10 mM GABA medium. Scale bar, 1 cm. (B) Quantification of hypocotyl growth in the indicated wild-type and *pop2* mutant plants. Results are the mean ± SE of measurements made on at least 62 hypocotyls distributed over three plates. (C–E) DIC microscopy analysis of hypocotyls. Typical images of wild-type (C) and *pop2-1* (D) 4-day-old dark-grown hypocotyls treated with 10 mM GABA are shown. A typical epidermal cell is pseudocolored in red. Scale bar, 100 µm. (E) Average epidermal cells size. Results are the mean ± SE of 21–26 cells derived from at least five hypocotyls. Student's *t*-test: ns, not significant; $^{***}P < 0.001$.

detected in the wild type regardless of whether they were grown in medium supplemented with 10 mM or no GABA (**Fig. 5C, E**).

To correlate hypocotyl growth inhibition with in planta GABA levels, we examined GABA levels in dark-grown hypocotyls of plants raised in control and 25 mM GABA-supplemented medium. The dark-grown hypocotyls of *pop2* plants were found to contain higher steady-state levels of GABA compared with wild-type plants, and the GABA levels increased to even higher levels when exposed to 25 mM exogenous GABA (**Supplementary Table S4**). Interestingly, dark-grown hypocotyls of even wild-type plants accumulated significant amounts of GABA when grown in 25 mM GABA-supplemented medium (**Supplementary Table S4**).

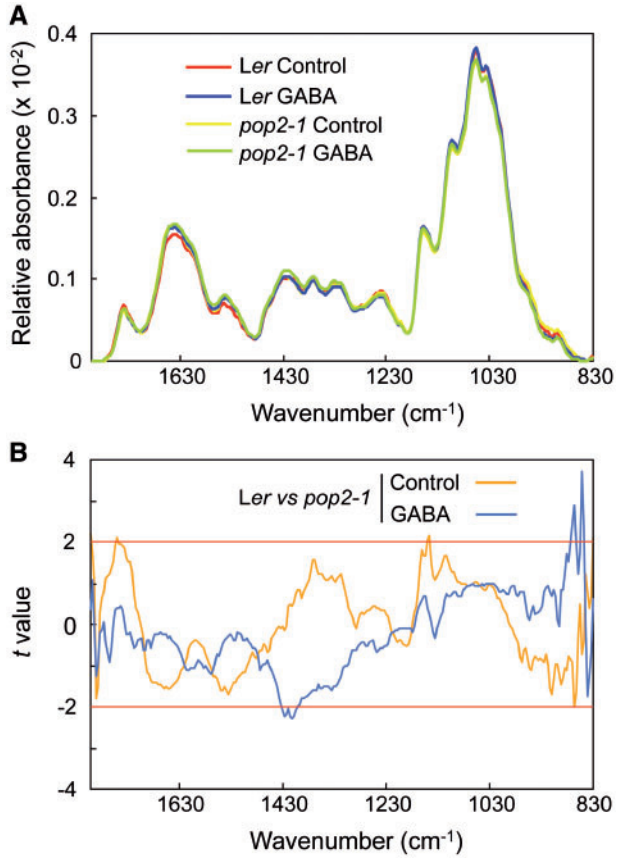


Fig. 7 Fourier transform-infrared (FT-IR) analysis of hypocotyl cell walls. (A) Average FT-IR spectra acquired from 19–21 hypocotyls of 4-day-old etiolated wild-type or *pop2-1* plants grown on agar medium supplemented with no (control) or 10 mM GABA. (B) Student's *t*-test values that assess the significance of differences between wild-type and *pop2-1* absorbances (*y*-axis) plotted, one each for comparisons between wild-type and *pop2-1* plants grown on medium supplemented with or without GABA (10 mM), against the wave numbers (*x*-axis). Red horizontal lines refer to the $P = 0.95$ significance threshold.

GABA accumulation does not alter hypocotyl cell wall composition

FT-IR microspectroscopy was used to investigate if changes in cell wall composition inhibited hypocotyl elongation in *pop2* mutants (**Fig. 7**). FT-IR analysis was carried out as previously reported (Mouille et al. 2003) on 4-day-old hypocotyls grown in the dark on GABA-supplemented agar medium. No significant difference in FT-IR spectra between the wild type and *pop2-1* mutants was observed in plants grown in control and 10 mM GABA conditions (**Fig. 7**). Similar results were also obtained when another *pop2* allele was tested; Col and *pop2-8* mutants treated with 10 mM GABA, as well as L*er* and Col ecotypes treated with 25 mM GABA, compared with control conditions, all produced nearly identical FT-IR spectra (data not shown). These findings indicate that the GABA inhibitory effect on hypocotyl growth was not due to modifications of cell wall composition; instead, it is likely to be a consequence of other defects in cell elongation such as cell wall loosening since a decrease in expression of at least three expansin genes was identified by microarray analysis in *pop2-1* plants treated with 1 mM GABA compared with the wild type (**Fig. 5**).

POP2 is widely expressed during plant development including in roots, leaves, pollen and pistils

The *POP2* gene encodes the first enzyme involved in GABA degradation (**Supplementary Fig. S1**) and was previously shown to be crucial for controlling GABA levels (Palanivelu et al. 2003, Renault et al. 2010). The cell type-specific expression pattern of *POP2* has not been determined previously and thus we could not correlate tissues that express *POP2* with the tissues affected in *pop2* mutants. Therefore, we investigated *POP2* expression during plant development using *pPOP2::GUS* reporter lines (Renault et al. 2010). We found that *POP2* was expressed in reproductive tissues (**Fig. 8A–C**). β-Glucuronidase (GUS) staining was detected in the stigma, style and abscission zones of siliques (**Fig. 8A**). Expression of *POP2* was also detected in stamens and pollen (**Fig. 8A–C**). Surprisingly, *POP2* expression was not observed in pollen tubes or the transmitting tract, a tissue type in which *pop2-1* tubes exhibited elongation defects (**Fig. 1A**). These results from phenotypic and expression analyses raise the possibility that loss of POP2 function in pollen, stigma and style (cell types in which *POP2* is expressed) manifests subsequently in *pop2* pollen tubes upon reaching the *pop2* transmitting tract after growing past the stigma and style. In vegetative tissues, the *POP2* promoter was active in dark-grown hypocotyls (**Fig. 8D**), leaves (**Fig. 8E, F**) and primary roots (**Fig. 8E, H**) in which various phenotypes manifested in *pop2* plants (**Figs. 6**, **2** and **3**, respectively). The expression pattern of *POP2* in elongating tissues such as primary root and hypocotyls was specific in that GUS staining was detected in the root tips and elongation zones but was absent in the division zone of the primary root (**Fig. 8H**). Even after an overnight incubation in the staining solution, GUS staining was not detected in the division zone of the primary root (data not shown), suggesting that either *POP2* is not expressed in this cell type or it is expressed at a level that is below the detection limits of this staining procedure. In hypocotyls, *POP2* expression was detected only in the base of hypocotyls (**Fig. 8D**) and GUS staining levels did not increase when plants were grown on plates supplemented with 1 mM GABA (data not shown). Intriguingly, *POP2* was also strongly expressed in guard cells (**Fig. 8F, G**), suggesting that GABA may be involved in stomata function and/or aperture regulation. Overall, *POP2* expression pattern analysis underscores the fact that there is a tight correlation between where phenotypes manifested (roots, hypocotyls, pollen and stigma/style) and where the *POP2* gene was expressed. These observations also raise the possibility that the GABA level must be controlled in plant growth zones.

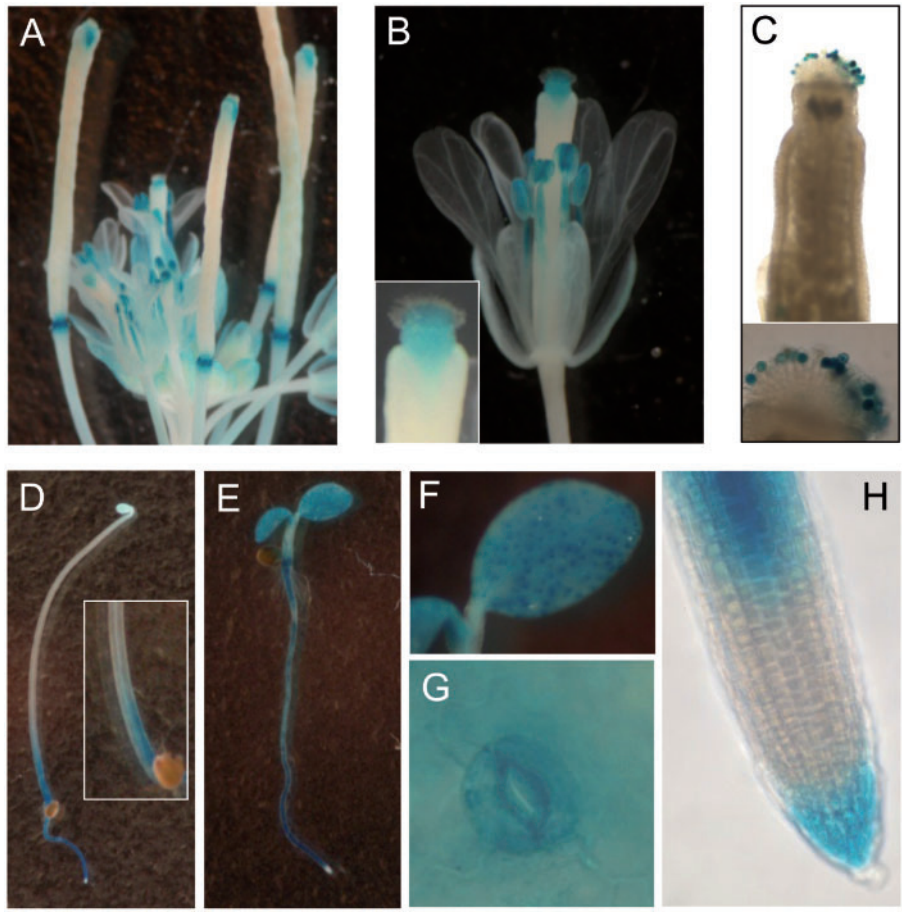


Fig. 8 Histochemical localization of *POP2* promoter activity. (A–H) In vivo analysis of the *POP2* expression pattern using *promoterPOP2::GUS* reporter lines. Each image shown is representative of the expression pattern consistently observed in three different reporter lines. GUS staining in (A) inflorescence, (B) flowers; inset, close-up view of stigma and style of a pistil, (C) wild-type pistil pollinated with *promoterPOP2::GUS* pollen; inset, *GUS* reporter pollen adhered to wild-type stigmata, (D) 4-day-old dark-grown hypocotyls. (E–H) GUS staining in 5-day-old seedlings, (E) whole seedling, (F) close-up view of staining in a cotyledon, (G) close-up view of GUS staining in stomata and (H) close-up view of staining in primary root apex.

Discussion

GABA alters reproductive and vegetative development

GABA and the genes involved in its metabolism are present in a wide range of organisms including bacteria (Lupo and Halpern 1970), fungi (Arst 1978), yeast (Reed 1950), algae (Tokunaga et al. 1979), plants (Steward et al. 1949) and mammals (Roberts and Frankel 1950). Hence, it is instructive to find common functions for GABA that have been retained, as well as specific functions that evolved in each kingdom. Consistent with this hypothesis, GABA has been shown to impact the development of several organisms such as bacteria (Foerster 1971, Chevrot et al. 2006), planktonic larvae (Morse et al. 1979) and mammals (Ganguly et al. 2001, Ge et al. 2005). In accordance with previous results obtained in tobacco and rice plants that accumulated GABA (Baum et al. 1996, Akama and Takaiwa 2007), our results showed that high GABA levels in Arabidopsis resulted in alteration of shoot and root development (**Figs. 2**, **3**). However, unlike the two previous studies (Baum et al. 1996, Akama and Takaiwa 2007), our strategy (i.e. impairment of GABA degradation) did not induce a severe disturbance of primary metabolism, especially of glutamate pools (**Supplementary Table S4**). These observations allow us to assume that the phenotypic effects we observed were primarily due to high GABA levels. To date, studies have mainly described the effects of GABA on pollen tube (Palanivelu et al. 2003) and stem growth (Baum et al. 1996, Kathiresan et al. 1998, Akama and Takaiwa, 2007); here, we report that GABA also affected hypocotyl and primary root growth (**Figs. 2**, **6**). Notably, in all instances, GABA was found to have an effect on cell elongation, suggesting that this non-protein amino acid has a specific developmental effect on cell growth. Several lines of evidence ascertain that responses to GABA observed in *pop2* mutants are the amplified responses occurring in a wild-type plant. First, very high GABA

levels can occur in plants under stressful conditions (up to 39 mM; Handa et al. 1983) and can concentrate in specific compartments such as the cell wall (Solomon and Oliver 2001). Hence, GABA levels as high as those recorded in GABA-treated *pop2* plants might, under certain circumstances, be observed in particular cellular compartments of a wild-type plant, thus triggering the same developmental responses. Secondly, in all organs studied, a similar trend was noticeable in growth inhibition, resulting from cell elongation defects, in wild-type plants, compared with *pop2* mutants, when treated with a higher GABA concentration. In this context, *pop2* mutants appeared as convenient GABA-overaccumulating systems, allowing us to trigger visible phenotypes, undetectable in a wild-type plant. Thirdly, microarray data from Roberts (2007) corroborate, at the molecular level, that *pop2* plants expressed amplified wild-type-like responses to GABA since a number of cell wall genes were down-regulated in wild-type plants treated with 10 mM GABA as observed in the *pop2-1* mutant treated with a lower GABA concentration (i.e. 1 mM).

High levels of GABA inhibit cell elongation

Gene expression changes identified by microarray experiments fit well with the inhibitory effect of GABA observed on at least three different elongating structures: pollen tubes, primary roots and hypocotyls (**Figs. 1**, **3**, **6**). Expression of genes encoding secreted and cell wall-related proteins decreased in vegetative tissues in response to GABA. Indeed, the impact of GABA on expression of these types of genes could affect cell elongation since cell growth is intimately coordinated with the deposition of new proteins, wall and membrane material (Hepler et al. 2001, Refrégier et al. 2004). Furthermore, cell elongation is driven by the equilibrium between turgor pressure and cell wall extensibility.

Microarray data provide evidence for GABA's role in cell elongation by acting on both cell turgor and wall extensibility. First, two aquaporin genes (*RD28* and *TIP2;2*) were found to be repressed by high GABA levels in *pop2-1* seedlings upon GABA exposure (**Fig. 5**). *RD28*, also termed *PIP2;3*, was initially identified among cDNAs responsive to desiccation (Yamaguchi-Shinozaki et al. 1992) and was subsequently shown to act as a plasma membrane water channel (Daniels et al. 1994). In contrast, TIP2;2 belongs to the family of tonoplast aquaporins implicated in vacuolar water transport and was previously found to be decreased in expression upon exposure to NaCl (Boursiac et al. 2005). Additionally, *POP2* was found to be strongly expressed in stomata, suggesting a function for GABA in guard cell metabolism (**Fig. 8**). Overall, these results indicate that GABA accumulation may impact water homeostasis in Arabidopsis and consequently cell turgor pressure. In support of this, the expression of two drought-responsive genes (*EARLY RESPONSIVE TO DEHYDRATION 5* and *DROUGHT-INDUCED 21*) was increased in the mutant after GABA exposure (**Supplementary Table S3** and **Supplementary Dataset S1**). Secondly, to withstand the mechanical force exerted by turgor pressure and enable cell elongation, the cell wall possesses remarkable properties of strength and pliability (Cosgrove 2000). Expansins are wall proteins involved in wall loosening that allows disentanglement of the polysaccharide matrix and thus cell elongation (Cosgrove 2000, Lee et al. 2001). Expression of three genes encoding expansins decreased in *pop2-1* seedlings upon exposure to GABA (*AtEXPA1*, *AtEXPA10* and *AtEXPA15*; **Fig. 5**), indicating that high GABA levels may affect cell wall loosening. Such a loosening defect could have consequences on organ growth since, for instance, *atexpa10* mutants exhibited decreased leaf area and petiole length (Cho and Cosgrove 2000). Cell wall composition was not altered, as revealed by FT-IR analysis (**Fig. 7**), raising the possibility that cell wall loosening defects may also cause the elongation deficiencies in *pop2* plants.

Above normal levels of GABA cause a decrease in expression of genes encoding secreted proteins

Previously, GABA has been shown to modulate the expression of *ACC synthase* (*ACS*) genes in sunflower (Kathiresan et al. 1997), of the *Nitrate Transporter 2* in *B. napus* (Beuve et al. 2004) and of *14-3-3* genes in Arabidopsis (Lancien and Roberts, 2006), revealing that GABA has potential signaling functions. To extend the gene expression analysis of GABA effects to the whole genome, we undertook a transcriptomic approach and used CATMA technology on vegetative tissues of Arabidopsis. Here we report an unexpected inhibitory effect of GABA accumulation on expression of genes encoding secreted proteins. Indeed, a significant number of genes whose expression is decreased in *pop2-1* seedlings, in the context of GABA accumulation, encode secreted proteins (**Fig. 5**). Thus, expression of genes encoding expansins, arabinogalactan proteins (AGPs) and proline-rich proteins were decreased in response to GABA accumulation in the mutant (**Fig. 5**). These results are consistent with those of Roberts (2007); using RNA isolated from wild-type Arabidopsis treated with exogenous GABA, the author identified 71 genes whose expression decreased, including a number of genes encoding cell wall proteins such as AGPs and hydroxyproline-rich glycoproteins (Roberts 2007). This study, along with our findings, shows that: (i) the category of genes whose expression decreases in the *pop2-1* mutant is similar to those that decrease in wild-type plants treated with exogenous GABA, suggesting that GABA accumulation accentuated deregulation of GABA-responsive genes; and (ii) GABA has a specific effect on genes encoding secreted proteins in different developmental conditions (mutant or wild-type seedlings).

It is unclear how GABA impacts the expression of genes encoding secreted proteins; however, evidence found in the literature underscores a link between GABA and the extracellular medium. First, as a neurotransmitter, GABA is released from vesicles into the synapse of mammals (Owens and Kriegstein, 2002); its neurotransmitter action is thus partly dependent on secretory activity. Secondly, in plants, it has been

demonstrated that GABA accumulation occurring under certain stressful conditions can lead to the appearance of GABA in the extracellular medium (Chung et al. 1992, Crawford et al. 1994, Solomon and Oliver 2001). For instance, GABA became the most abundant amino acid in the tomato apoplast during pathogen infection, with concentrations reaching up to 2–3 mM (Solomon and Oliver 2001). Three hypotheses can be proposed: (i) GABA could act on transcriptional factors by activating a signal transduction pathway; (ii) GABA accumulation in the apoplast could lead to negative feedback regulation of the secretory pathway; or (iii) as shown in neurons (Bouzigues et al. 2007), GABA could have a trophic action on cell development.

GABA accumulation: a role in growth control?

GABA accumulates in plants in response to a wide range of environmental cues (Kinnersley and Turano 2000) rising sometimes to very high levels. For instance, tobacco cells adapted to NaCl contained a concentration of 6.72 mM GABA (Binzel et al. 1987), and tomato cells adapted to water stress had a GABA concentration of up to 39 mM (Handa et al. 1983). These observations raise the question of the role and consequences of GABA accumulation. Previously, we described the hypersensitivity to salt stress of the *pop2-1* mutant, especially at the root growth level, indicating that GABA degradation was a determinant of salt tolerance (Renault et al. 2010). Furthermore, we showed that the *POP2* expression pattern was tightly reconfigured during salt treatment in the primary root apex (Renault et al. 2010). These data indicate that control of GABA levels is important in the growing part of roots under stressful conditions. Taking together these observations and the results described in this study, we speculate that GABA accumulation could be responsible for a decrease in cell elongation under stressful conditions and that GABA could be viewed as a growth control factor. Consistently, Park et al. (2010) recently demonstrated, using either a *Pseudomonas syringae* strain deficient for three GABA transaminases (Δ*gabT1*/*T2*/*T3* mutant) or the *pop2-1* mutant, that high GABA levels could significantly inhibit growth of the bacteria. More interestingly, the authors showed that the growth restriction in the Δ*gabT1*/*T2*/*T3* strain upon GABA exposure was accompanied by a decrease in expression of *hrpL* and *avrPto* genes which encode, respectively, an alternative sigma factor and effector associated with the type III secretion system (Park et al. 2010). These results support the hypothesis of an inhibitory effect of GABA accumulation on cell growth through a down-regulation of genes of the secretory pathway and suggest that GABA effects are conserved in organisms.

However, since growth control involves a wide array of mechanisms, it would be surprising if GABA functioned solely to trigger the developmental changes observed. In contrast, it is more likely that GABA acts in concert with phytohormones. In support of such a hypothesis, we showed that the MapMan term 'hormone metabolism' was significantly enriched among genes increased in expression in the *pop2-1* mutant upon exposure to GABA (**Table 2**); furthermore, GABA is known to promote ethylene synthesis in sunflower by inducing *ACS* mRNA accumulation (Kathiresan et al. 1997) and was found to cause a decrease in expression of *14-3-3* genes in a Ca^{2+}-, ethylene- and ABA-dependent manner (Lancien and Roberts 2006). Since we did not identify *ACS* genes in our microarray data set, ABA is more likely to modulate GABA effects. Intriguingly, *AGP30*, the most repressed gene in *pop2-1* seedlings upon GABA treatment, is involved in primary root regeneration and has been shown to accelerate ABA responses in roots (van Hengel and Roberts 2003, van Hengel et al. 2004).

Materials and Methods

Plant materials and growth conditions

Seeds of *pop2* mutants used in this study were described previously {*pop2-1*, L*er* background (Palanivelu et al. 2003); *pop2-3*, Ws background (Palanivelu et al. 2003); and *pop2-8*, Col background [*gabat1-1* in Miyashita and Good (2008) but renamed *pop2-8* in this study as *pop2-4* to *pop2-7* were published (Ludewig et al. 2008)]}. Accordingly, L*er*, Ws and Col were used as wild-type controls for *pop2-1*, *pop2-3* and *pop2-8*, respectively. For floral analysis, wild-type and *pop2-1* mutants were grown as described (Palanivelu et al. 2003). For vegetative tissue analysis, plants were grown on agar plates as reported (Renault et al. 2010). GABA treatments were carried out by supplementing freshly autoclaved agar medium with filter-sterilized (0.22 μm filter; Millex®-GS, Millipore) 2 M stock solution of GABA dissolved in water. The age of plants was determined starting from the time when stratification was completed.

Microscopy

For aniline blue staining, pistils were fixed in 1 : 3 acetic acid : ethanol at room temperature for at least 2 h, rehydrated in an ethanol series, then suspended in distilled water. Subsequently, pistils were softened in 8 M NaOH overnight and washed in water for 10 min. Pistils were then stained with decolorized aniline blue 0.1% (w/v) for at least 2 h in dark conditions, mounted on slides and observed using the DAPI (4′,6-diamidino-2-phenylindole) filter with a Zeiss Axioscope. Samples were viewed with a 63× (numerical aperture 1.2) water-corrected objective on a Zeiss confocal microscope. Siliques were inspected for unfertilized ovules and reduced seed set after removing ovary walls under a dissecting scope (Stemi 2000, Carl Zeiss). For confocal imaging, roots were cut using a razor blade, stained with a 10 μg ml^{-1} propidium iodide (Sigma, P4170) solution for 1 min, quickly rinsed with ultrapure water and mounted on a slide in water. Observations were carried out on a Leica SP5 confocal microscope using a 20× objective and 488/570–700 nm excitation/emission wavelengths. Images of the beginning of the root hair zone alone were acquired. To determine dark-grown hypocotyl cells size,

etiolated seedlings were fixed in ethanol/acetic acid solution (9/1) for 5 h and rehydrated in 90 and 70% ethanol solutions. Prior to observing under a microscope, samples were cleared using a chloral hydrate/water/glycerol solution (4/2/1, w/v/v) and mounted on a slide in the clearing solution. Epidermal cells were observed at half points of hypocotyls using DIC microscopy and a 10× objective.

Photosynthetic pigment analysis

Aerial parts of 14-day-old seedlings were harvested, snap-frozen in liquid nitrogen and homogenized using a mixer mill (MM 400, Retsch) with 4 mm steel balls for 30 s at 30 s^{-1} frequency under frozen conditions. Photosynthetic pigments were extracted with 80% acetone at 8°C for 1 h under 1,000 r.p.m. agitation; following centrifugation at 13,000 × *g* for 10 min, supernatants were recovered. Absorbance at 663, 647 and 470 nm was recorded, and Chl and carotenoid contents were then calculated according to Lichtenthaler's equations (Lichtenthaler 1987).

Root and hypocotyl length measurements

To determine the effect of GABA on primary root growth, mutant and wild-type seedlings were germinated on agar plates. Four days later, seedlings [Boyes' stage 1.0 (Boyes et al. 2001)] were transferred to GABA-supplemented plates and the primary root apex at the time of transfer was marked. Plates were photographed 6 d after transfer and root elongation was measured using the ImageJ software (http://rsbweb.nih.gov/ij/). For hypocotyl length measurements, sterilized seeds were sown on agar plates and stratified for 2 d to synchronize germination. Seeds were then treated with white fluorescent light (100 µmol m^{-2} s^{-1}) for 4 h at 22°C to stimulate germination. Plates were subsequently wrapped with aluminum foil and photographed 4 d later. Time 0 was defined as the end of stratification. Hypocotyl length was measured using the ImageJ software (http://rsbweb.nih.gov/ij/).

Fourier transform-infrared microspectroscopy

FT-IR analyses were carried out at the 'Plateau Technique de Chimie du Végétal' (OC028 INRA; Institut Jean-Pierre Bourgin UMR1318 INRA/AgroParisTech). For each genotype/treatment, five seedlings of four independent biological replicates were collected (i.e. a total of 20 seedlings). Seedlings were squashed between two BaF_2 windows and rinsed thoroughly in distilled water for 2 min. The samples were then dried on the window at 37°C for 20 min. An area of 50 µm × 50 µm in the middle area of each hypocotyl on the side of the central cylinder was selected for FT-IR microspectroscopy. A Thermo-Nicolet Nexus spectrometer equipped with a Continuµm microscope accessory (Thermo scientific) was used. Fifty interferograms were collected in transmission mode, with 8 cm^{-1} resolution, and co-added to improve the signal-to-noise ratio. The collected spectra were baseline corrected and normalized as described in Mouille et al. (2003). Raw data are available upon request.

Determination of levels of GABA and other amino acids

Plant samples were harvested, rinsed three times in ultrapure water, quickly blotted and snap-frozen in liquid nitrogen. Samples were freeze-dried and then homogenized with 4 mm steel balls for 1 min at 30 s^{-1} frequency. Amino acid extraction and analysis were performed as described in Renault et al. (2010).

RNA isolation and qRT-PCR analysis

Total RNA was isolated from 30 mg of fresh material using the SV Total RNA Isolation Kit (Promega Corporation) following the manufacturer's protocol. Samples were treated with DNase I using the TURBO DNA*free* kit (Applied Biosystems). qRT-PCR analysis was carried out as reported (Renault et al. 2010). The *PP2AA3* gene was chosen as an internal reference for data normalization since it showed stable and constitutive expression across all conditions according to the microarray results (**Supplementary Dataset S1**). Primer pairs used for PCR amplification are listed in **Supplementary Table S5**; PCR efficiency of primer pairs ranged from 92 to 99%.

Transcriptome studies and statistical analysis of microarray data

Microarray analysis was carried out at the 'Unité de Recherche en Génomique Végétale' (Evry, France), using the CATMA arrays containing 24,576 gene-specific tags (GSTs) corresponding to 22,089 genes from Arabidopsis (Crowe et al. 2003, Hilson et al. 2004). Two independent biological replicates were analyzed. For each biological replicate, RNA samples were obtained from 30 plants. Total RNA was extracted using the SV Total RNA Isolation Kit (Promega Corporation) and subsequently treated with DNase I using the TURBO DNA*free* kit (Applied Biosystems). For each comparison, one technical replicate with fluorochrome reversal was performed for each biological replicate (i.e. a total of four hybridizations per comparison). The labeling of cRNAs with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products), the hybridization to the slides and the scanning were performed as described (Lurin et al. 2004).

Experiments were designed with the statistics group of the 'Unité de Recherche en Génomique Végétale'. Normalization and statistical analysis were based on two dye swaps (i.e. four arrays, each containing 24,576 GSTs and 384 controls) as described in Gagnot et al. (2007). To determine differentially expressed genes, we performed a paired *t*-test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw *P*-values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate (with a type I error equal to 5%), in order to keep a strong control of the false positives in a multiple-comparison context (Ge et al. 2003). As described in Gagnot et al. (2007), genes with

a Bonferroni *P*-value $\leq$0.05 were considered as being differentially expressed.

Microarray data from this analysis were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; GEO accession number: GSE11129) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: AU05-03_GABA) according to the 'Minimum Information About a Microarray Experiment' standards.

Histochemical localization of *POP2* promoter activity

The expression pattern of the *POP2* gene was investigated using *pPOP2::GUS* transgenic lines (L*er* background) and a published GUS staining protocol (Renault et al. 2010). For shoots and roots, 5-day-old seedlings grown on agar plates were used. GUS staining of floral tissues was carried out on inflorescence from 60-day-old soil-grown plants. For analysis of *POP2* expression pattern in pollen, pistils of 60-day-old wild-type plants were pollinated with pollen from *pPOP2::GUS* lines before GUS staining. Four-day-old dark-grown seedlings were used to investigate the *POP2* expression pattern in hypocotyls.

Supplementary data

Supplementary data are available at PCP online.

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